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Effects of probiotics, prebiotics, and synbiotics on mineral metabolism in ovariectomized rats — impact of bacterial mass, intestinal absorptive area and reduction of bone turn-over

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ABSTRACT

Background: Defined prebiotics were shown to improve calcium balance and diminish bone loss. However, the effect of their combination with probiotics on gut ecology and bone metabolism has not yet been studied. We investigated whether the combination of a probiotic with a defined microbial strain results in improved bone mineralization, and whether this effect is associated with changes in gut ecology.
Methods: Eighty ovariectomized adult rats were allocated to five groups: group 1, sham-operated (SHAM); group 2–5, ovariectomized (OVX). Semipurified diets containing 0.7% calcium and 0.5% phosphorus were fed for 16 weeks, group 1 and group 2 got no supplements, group 3 (PRO) was supplemented with a potential probiotic (*Lactobacillus acidophilus NCC90*), group 4 (PRE) with prebiotics (oligofructose + acacia gum) and group 5 (SYN) with synbiotics (probiotics + prebiotics).
Results: Ovariectomy increased body weight and reduced bone weight, content of calcium, phosphorus and ash of bone, bone alkaline phosphatase (BAP), and bone structure. This was indicated by lower trabecular bone area, trabecular perimeter, and connectivity but higher epiphyseal breadth. Ovariectomy elevated the jejunal pH. The probiotic alone did not significantly affect bone mineralization and gut ecology. Rats on prebiotics had significantly higher amounts of cecal contents and lower pH in cecal and colonic contents. Their calcium balance tended to be increased ($p < 0.1$). Synbiotics reduced pH in different intestinal segments, significantly in cecum. They stimulated most the colonic absorption surface as indicated by colon weight. Only feeding synbiotics significantly prevented OVX-induced loss of calcium content in lumbar vertebrae (mg) with final values (mean ± SD) of 44.44 ± 2.94 (SHAM), 41.20 ± 4.59 (OVX), 41.63 ± 3.78 (PRO), 43.42 ± 3.07 (PRE), and 44.68 ± 2.28 (SYN). This effect was associated with higher counts of bifidobacteria in the short-term and *Bacteroides* in the long-term, and with a tendency for lower BAP with 128.7 ± 28.5 U/L vs. 155.3 ± 28.1 U/L in OVX ($p < 0.1$).
Conclusion: SYN exerted a synergistic effect on bone mineralization, presumably due to changes in gut microbiota and ecology associated with large bowel digesta weight (most likely reflecting microbial mass) and with large bowel weight (reflecting absorptive area), while bone turnover tended to be reduced as indicated by BAP.

Abbreviations: ANOVA, analysis of variance; Ar.MS, total area of mineralized structure; BAP, bone specific alkaline phosphatase; BMC, bone mineral content; BPm, trabecular bone perimeter; CFU, colony-forming unit; C.Th, cortical bone thickness; EpB, epiphyseal breadth; FOS, fructooligosaccharides; GOS, galactooligosaccharides; OVX, ovariectomized; PRE, prebiotics; PRO, probiotics; S.Ar, trabecular skeleton area; SCFA, short-chain fatty acid; SD, standard deviation; SHAM, sham-operated; SYN, synbiotics; T.Ar, bone tissue area; Tb.Ar, trabecular bone area; Tb.BrP, trabecular branch points; Tb.D, trabecular density; Tb.N, trabecular number; Tb.Pm, trabecular perimeter; Tb.Th, mean trabecular thickness; TBPf, trabecular bone pattern factor; TNF, tumor necrosis factor.

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1. Introduction

Osteoporosis is a multifactorial bone disease with increasing prevalence and importance, also because of ascending life expectancy. In the United States of America prevalence of osteoporosis is 10.3% and prevalence of low bone mass is 43.9% in adults aged 50 years and older [1]. For prevention and treatment of osteoporosis nutrition is one factor to be considered. Older adults (> 50 years) are recommended a diet that supplies adequate amounts of calcium (1000 mg/day for men and 1200 mg/day for women) [2]. Enhancers of bioavailability in the diet are a further approach to increase calcium absorption and bone mineral content (BMC), and approaches for improving bone health by bioactive foods and ingredients are discussed [3].

Prebiotics selectively stimulate the growth and/or activity of specific bacteria, mainly bifidobacteria and lactobacilli and by this deploy a microbiota-mediated health effect [4–8]. They include inulin-type fructans, fructooligosaccharides (FOS), galactooligosaccharides (GOS), as well as lactulose, sugar alcohols, resistant starch and complex polysaccharides such as acacia gum. Several non-digestible substrates, predominantly carbohydrates, are fermented in the large intestine. Among others, prebiotics increased the absorption of minerals and trace elements that have an impact on the mineralization of bone, increased BMC and bone trabecular structure, or reduced estrogen-deficiency-induced bone loss in the rat [for review see Refs. 7,9–14]. Inulin-type fructans also increased calcium absorption and bone mineralization in young healthy humans [15–17]. Probiotics are viable, defined microorganisms, in many cases bifidobacteria and lactobacilli, which alter the composition and/or activity of the microbiota of the host, provided that they have been ingested in sufficient number and survived the gastrointestinal transit. Thereby probiotics exert beneficial effects on the host's health and well-being [5,6]. Although beneficial effects of probiotics have been reviewed with respect to various diseases [6,18,19,20], there are only few studies on effects of probiotics on bone mineralization or osteoporosis [21]. A synbiotic is a combination of a specific prebiotic with a defined probiotic which exerts a beneficial effect beyond their individual effect, presumably by improving the gastro-intestinal survival and activity of beneficial microbes [5,20]. The investigated bacteria mainly belonged to the genus bifidobacteria or lactobacilli. Greater bone density in germ-free mice compared to conventional animals [22] suggests also a role of gut microbiota in bone mineralization. Accordingly, specific bacterial strains may display a probiotic potential with respect to bone preservation [12], but so far little is known about probiotics or synbiotics and their bone mineral preserving effect.

We have shown before that the beneficial effect of oligofructose on bone mineral content and trabecular structure depended on its amount in the diet, on the amount of calcium in the background diet, on the duration of intervention and on the investigated skeletal site [23]. We had observed stimulating effects of oligofructose on calcium absorption after 8 and 16 weeks but not after 4 weeks, and on calcium content and trabecular area in bone. The effects were significant if the dietary calcium was high (1%), but was less pronounced if the background diet was 0.5%. We now chose a dietary calcium content of 0.7% and phosphorus content of 0.5%. Thus the mineral contents were higher than the recommended amount for adult rats for maintenance of 0.5% for calcium and 0.3% for phosphorus, but their Ca:P ratio remained in the same range. In the present study we tested a potential probiotic strain (*Lactobacillus acidophilus NCC90*) and a potential prebiotic substance (oligofructose + acacia gum) for their short-term and long-term effect on bone mineralization of femora and lumbar vertebrae. We compared these supplements with the intake of the potential synbiotic (*L. acidophilus NCC90* plus oligofructose + acacia gum). The choice of the lactobacilli was based on a series of in vitro experiments showing enhancement of calcium absorption using human intestinal cell lines in which the *L. acidophilus* was the most promising bacterium among the ones tested (data not shown). Acacia gum was added, since it had been shown to be bifidogenic [24,25], to induce lactobacilli [25], and to be better tolerated than oligofructose alone [24,26]. To describe possible underlying mechanisms, we assessed parameters of gut ecology and performed microbiological analyses of feces.

2. Materials and methods

2.1. Study design, experimental groups, animals and diets

This experiment was approved by the German Institutional Animal Experiment Committee (Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein). The study was performed with eighty virgin female Fisher-344 rats. Weanling rats were purchased from Harlan/Winkelmann, (Borchen, Germany). Until the age of 19 weeks, rats were fed ad libitum with a commercial standard rat diet. Two weeks before starting the intervention (study week −2) feed was switched from standard diet to a semipurified diet that was used as a control diet in studies with adult rats in our laboratory [23] providing all nutrients in sufficient amounts. Thereafter, at the age of 21 weeks (study week 0) rats were divided into five groups of 16 animals (Table 1). They were matched by body weight, and were sham-operated (SHAM) or ovariectomized (OVX, PRO, PRE and SYN).

Ovariectomy was done under anesthesia with intraperitoneal injection of xylazinhydrochloride/ketaminhydrochloride (Rompun®/Ketavet®). The intervention period of 16 weeks started by feeding 8.5 g per day of the semipurified diet (Table 2) to SHAM and OVX, or the semipurified diet supplemented with a probiotic (PRO), or with 2.5% of a prebiotic at the expense of corn starch (PRE) or with both as a synbiotic (SYN). The potential probiotic (*L. acidophilus NCC90*) was added to an acidified milk, both provided by Nestlé Research Center, Lausanne, Switzerland. The probiotic was supplied as fresh frozen bacteria culture as sets of vials stored in liquid nitrogen. Each vial contained sufficient bacteria for the preparation of one batch of probiotic feeding for 1 week. For this the acidified milk powder was reconstituted with demineralized water. One vial of thawed probiotic was freshly added to the milk slurry every week to get a concentration of $1–5 \times 10^8$ cfu per 100 g. One gram of the probiotic acidified milk was given each day, which was equivalent to $1–5 \times 10^6$ cfu per rat and day. The probiotic "yoghurt" was consumed by the groups PRO and SYN on top of their feed.

The prebiotic was a 50% mixture of Raftilose P95®, (Orafti, Belgium) and acacia gum (Fibergum®, CNI, France). The prebiotic mixture and the probiotic were provided by Nestlé Research Center, Lausanne, Switzerland. The form of calcium used in the diets was tri-calcium dicitrate tetrahydrate ($C_{12}H_{10}Ca_3O_{14}\cdot 4H_20$). To guarantee strict pair feeding the reservoirs were filled twice weekly with 8.5 g/day. In earlier studies this amount represented a restricted feed intake for about 30% and an ad libitum intake for 70% of the animals [23]. Again in this experiment, all rats had consumed all their feed after 3 or 4 days. There were no back-weights of feed. Rats had free access to the feed reservoir,

Table 1
Experimental groups.

		Dietary components				Matching	
		Ca	P	PRE	PRO	Body wt. (g)	
Group	OP	(g/kg diet)				Week 0	Global p
SHAM (Control 1)	Sham	7	5	0	No	166.7 ± 7.2	ns
OVX (Control 2)	OVX	7	5	0	No	169.6 ± 7.6	
PRO	OVX	7	5	0	Yes	169.1 ± 8.0	
PRE	OVX	7	5	25	No	169.9 ± 7.6	
SYN	OVX	7	5	25	Yes	170.7 ± 6.8	

Means ± SD, n = 15–16, p-value from ANOVA. SHAM = Sham-operation; OVX = ovariectomy;
PRO = OVX + Probiotics (1–5*10^6 cfu of *L. acidophilus NCC90/g*) mixed with yoghurt;
PRE = OVX + Prebiotics (fructooligosaccharide, Raftilose P95® + acacia gum (50:50);
SYN = OVX + Synbiotics (PRO + PRE).

Table 2
Composition of the semi-purified diet: constant components.

Component	(g/kg diet)
Casein	160
Soy oil	50
Cellulose	50
Calcium-free mineral premix[a]	118
Calcium premix[b]	60
Vitamin premix[c]	10
Oligofructose premix[d]	100
Corn starch	452
Total	1000

[a] Contained minerals equivalent to (g/kg diet): $MgHPO_4$*$3H_2O$ (5.31); $NaHPO_4$ *$2H_2O$ (4.22); K_2HPO_4 (13.53); KCl (2.52); $MgSO_4$*$7H_2O$ (1.59); (mg/kg diet): $C_6H_8O_7NFe$ (338); $MnSO_4$.*H_2O (162); $CuSO_4$ *$5H_2O$ (40), KI (1.56); NaF (10.2); NH_4 Al $(SO_4)_2$ *$12H_2O$ (3.64); $ZnSO_4$ *$7H_2O$ (221); Na_2SeO_3 *$5H_2O$ (0.23); KBr (20.1); $NiSO_4$ *$6H_2O$ (8.5); $CoSO_4$ *$7H_2O$ (5.06); Na_2MoO_4 *$2H_2O$ (5.06); $NaAsO_2$ (0.25); $Na_2B_4O_7$ *$10H_2O$ (5.06), $CrCl_3$ *$6H_2O$ (0.89). All mineral components were from Merck (Darmstadt, Germany).

[b] Contained calcium from $C_{12}H_{10}Ca_3O_{14}$ *$4H_20$.

[c] Contained vitamins equivalent to (mg/kg diet): vitamin A (500 I.E./mg), 9.1; vitamin D_3 (500 I.E./mg), 2.33; vitamin E (50%), 68.4; vitamin K_3 (100%) 11.4; choline (50%) 2280; folic acid (100%), 1.2; nicotinic acid (98%), 23.3; pantothenic acid (98%), 9.3; riboflavin (96%), 3.6; thiamin (98%) 4.7; pyridoxine (98%) 7.0; cobalamin (0.10%), 57.0; biotin (2%), 22.8. Vitamins were purchased from Synopharm (Germany) except vitamins K_3 (Sigma, Deisenheim, Germany), A, C, and E (Deutsche Vilomix, Hessisch Oldendorf, Germany).

[d] Contained 25 g oligofructose and 75 g corn starch.

which were inspected twice daily, and to demineralized water. Animals were housed individually in stainless-steel wire mesh cages in a room with air condition, relative humidity (60–70%), controlled temperature (22–25 °C), and a 12-hour dark/light cycle. After 16 weeks animals were sacrificed by desanguination under anesthesia with intraperitoneal injection of xylazinhydrochloride/ketaminhydrochloride (Rompun®/Ketavet®). Blood was taken and the intestine, femora, tibiae and lumbar vertebrae were removed.

2.2. Mineral balances, chemical analyses

Individual metabolic balances were performed with separate collection of urine and feces over 7 days at week 6 or 16 to investigate the short and long-term effect of dietary probiotics, prebiotics, and synbiotics. Urinary samples were ashed at 450 °C in a muffle furnace for 16 h and solubilized in 20% hydrochloric acid (Merck, Darmstadt, Germany) and 8% nitric acid (Merck, Darmstadt, Germany). Feces were solubilized in 65% nitric acid (Merck, Darmstadt, Germany) and 70% perchloric acid (Merck, Darmstadt, Germany). Calcium in urine, feces and bone samples was measured by atomic absorption spectrometry (Perkin-Elmer 1100) using an air/acetylene flame at 2300 °C. Phosphorus in urine and feces was analyzed as inorganic phosphorus with an automatic analyzer (CobasBio, Hoffmann La-Roche, Basel, Switzerland) using the kit Unimate 7 PHOS (Hoffmann La-Roche, Basel, Switzerland). Phosphorus in bones was measured in the solutions used for the measurement of calcium. Apparent absorption and retention of minerals (mg/day) were calculated: apparent mineral absorption (mg/7 days) = mineral intake (mg/7 days) − fecal mineral excretion (mg/7 days); mineral retention (mg/7 days) = mineral intake (mg/7 days) − [fecal mineral excretion (mg/7 days) + urinary mineral excretion (mg/7 days)]. Activity of bone specific alkaline phosphatase (BAP) was analyzed after lectin precipitation with Unimate 3 ALP (Boehringer, Mannheim, Germany) in a kinetic colorimetric test using an automatic analyzer (CobasBio, Hoffmann La-Roche, Basel, Switzerland).

2.3. Bone mineral content

Lumbar vertebrae (1 and 4) and right femora were collected and adherent soft tissue was removed carefully before weighing. Dry matter was gained after drying at 105 °C for 4 h. The dried samples were ashed at 450 °C for 16 h. The ash was weighed and dissolved in hydrochloric acid (6.0 mol/L) and calcium content of LV and right femora was measured by atomic absorption spectrometry (Perkin-Elmer 1100).

2.4. Microradiography/histomorphometry

This method had been applied in comparable experiments for investigating calcium bioavailability from different diets and was described in more detail elsewhere [23]. In short, the proximal third of the left non-decalcified tibiae was embedded in methyl methacrylate, sawn and polished. Contact microradiographs (Fixitron X-Ray Systems; Hewlett-Packard, Manville, USA) were made under constant conditions (18 kV, 5 mA, 8 min exposure time) and documented on high-resolution plates (Microchrome Technology, San Jose, USA). The quantitative assessment of trabecular structure was determined by computer-supported image analysis of video-scanned microradiographs (TK 1280 E; JVC, Berlin, Germany) connected to a light microscope (Mikrophot FXA; Nikon, Düsseldorf, Germany). An example of the obtained images is shown in Fig. 1. The following histomorphometric parameters were measured: trabecular bone area (Tb.Ar/T.Ar, %), trabecular number (Tb.N, *n*), trabecular perimeter (Tb.Pm, mm), mean trabecular thickness (Tb.Th, μm), and trabecular bone pattern factor (TBPf, mm^{-1}), which indicates a loss of connectivity, cortical bone thickness (C.Th, μm), epiphyseal breadth (EpB, μm), and number of trabecular branch points (Tb.BP, *n*). All analyses were done blinded and in duplicate.

2.5. Gut ecology

Gut segments were weighed before and after rinsing with demineralized water and the pH of digesta was measured after dilution of digesta samples with demineralized water. Microbiological analyses of the endogenous populations of lactobacilli, *Bacteroides*, *Enterobacteriaceae*, and bifidobacteria were counted at week 6 and 16, when spontaneously excreted fecal samples were collected from the anus of each animal. The samples were immediately diluted with Ringer solution and prepared to be frozen in liquid nitrogen and stored at −80 °C until analysis. For bacterial enumeration the tubes were rapidly thawed. Hundred fold serial dilutions were performed in pre-reduced Ringer solution containing 0.5% of cysteine. Petri dishes of various media were inoculated and incubated for 48 h at 37 °C in anaerobic atmosphere using AnaeroGen (Oxoid, England), except for *Enterobacteriaceae*, which were incubated for 24 h at 37 °C in aerobic atmosphere. Bacteria were detected on selective or half-selective media as follows: *Enterobacteriaceae* on Drigalski medium (Sanofi Diagnostics Pasteur, France), bifidobacteria on Eugon Tomato medium (Wadsworth Anaerobic Bacteriology Manual, V. Suter, D. Citron and S. Finegold Third edition), lactobacilli in MRS (Difco, MI, USA) with antibiotics (phosphomycine (79.5 mg/L) + sulfamethoxazole (0.93 mg/L) + trimethoprime (5 mg/L)), *Bacteroides* on Schaedler Neo-Vanco medium (BioMérieux, Marcy-l'Etoile, France). After incubation, the colonies were counted and further identified if necessary. Lactobacilli and bifidobacteria strains were identified by microscopy,

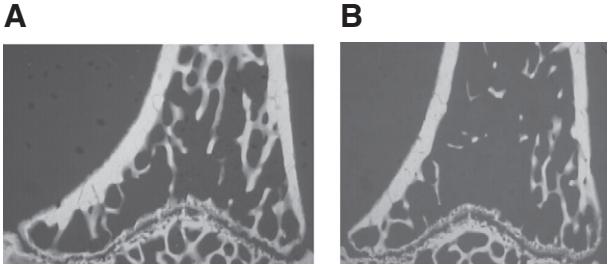


Fig. 1. Representative microradiographs of the tibia of rats after sham-operation (A) or ovariectomy (B).

Table 3
Effect of ovariectomy on body weight and uterus weight, bone and intestinal parameters, as assessed after 16 weeks intervention.

	SHAM	OVX	*p*
Weight (g)			
Body	178.47 ± 4.52	184.39 ± 3.54	<0.05
Uterus	0.326 ± 0.050	0.085 ± 0.020	<0.05
Bone weight (mg)			
Lumbar vertebrae	264.7 ± 24.4	240.3 ± 29.2	<0.05
Calcium content (mg)			
Lumbar vertebrae	44.44 ± 2.94	41.20 ± 4.59	<0.05
Femora	88.94 ± 3.40	84.03 ± 8.13	<0.10
Phosphorus content (mg)			
Lumbar vertebrae	21.76 ± 2.78	19.41 ± 2.38	<0.05
Femora	41.33 ± 2.71	39.25 ± 4.17	<0.10
Ash content (mg)			
Lumbar vertebrae	124.3 ± 8.4	116.3 ± 13.2	<0.05
Femora	245.4 ± 10.05	231.2 ± 20.8	<0.05
Osteoblasts activity			
BAP (U/L)	177.99 ± 30.48	155.32 ± 28.14	<0.05
Bone quality			
Tb.Ar/T.Ar (%)	15.44 ± 2.94	12.25 ± 2.81	<0.05
Tb.N (*n*)	27.94 ± 5.62	28.20 ± 10.26	ns
Tb.Pm (mm)	34.58 ± 5.98	29.52 ± 5.64	<0.05
Tb.Th (mm)	0.020 ± 0.002	0.020 ± 0.002	ns
TBPF (mm^{-1})	19.96 ± 4.96	25.32 ± 4.40	<0.05
C.Th (mm)	0.34 ± 0.06	0.38 ± 0.16	ns
EpB (mm)	0.079 ± 0.013	0.093 ± 0.012	<0.05
Tb.BP (*n*)	152.81 ± 28.45	134.07 ± 27.48	ns
Intestinal pH			
Jejunum	7.12 ± 0.60	7.69 ± 0.45	<0.05
Cecum	7.93 ± 0.23	8.00 ± 0.29	ns
Colon	8.24 ± 0.23	8.25 ± 0.29	ns

Means ± SD; *p*-values from t-tests; ns = not significant; SHAM = sham operated control rats ($n = 16$), OVX = ovariectomized rats ($n = 16$).

and biochemically using the API gallery system (BioMérieux), API 50 CHL gallery for lactobacilli, and API ID 32 A gallery for bifidobacteria respectively. Bacterial counts are expressed as log10 colony-forming units (CFUs) per gram of fresh fecal sample, with detection limit at 3.30 cfu/g.

2.6. Statistics

For statistical analysis the program STATGRAPHICS Plus Version 4.1 was used. Effects of ovariectomy on body weight, bone and intestinal parameters and bone histomorphometry were tested by comparing the sham-operated group with the ovariectomized control group (OVX) using independent *t*-tests. Differences between the interventional groups (PRO, PRE and SYN) and the ovariectomized control group (OVX) were tested using the Dunnett's procedure. Pearson correlation coefficients between bone mineral and intestinal variables were assessed on the four ovariectomized groups. Differences were regarded as significant if p was <0.05 and were regarded as a notable tendency if p was <0.10.

3. Results

3.1. Effect of ovariectomy

Completeness of ovariectomy was confirmed by absence of ovarian tissue and significantly lower uterus weight compared to intact animals (Table 3). Ovariectomy compared to sham-operation caused a significant increase in body weight, as well as significant losses of bone weight and calcium, phosphorus and ash content in lumbar vertebrae. In femora ash content was also significantly lower, whereas the reduction of calcium and phosphorus content tended to be lower ($p < 0.10$, Table 3). BAP was also significantly lower in ovariectomized compared to sham-operated rats. Ovariectomy compared to sham-operation caused a significant loss of trabecular bone (Table 3). Trabecular bone area (Tb.Ar/T.Ar) and trabecular perimeter (Tb.Pm) were significantly lower, while lack of connectivity (TBPf) and epiphyseal breadth (EpB) were significantly higher. The intestinal milieu was also affected by ovariectomy. The pH value in jejunum increased compared to sham operation (Table 3).

3.2. Effects of probiotics

Probiotics-fed ovariectomized rats (PRO) compared to ovariectomized control rats (OVX) did not show significant differences in calcium and phosphorus absorption or retention, either at week six (not shown) or 16 (Fig. 2). Bone mineral content in femora and lumbar vertebrae (Fig. 3) and bone structure (Table 4), were not affected by *L. acidophilus NCC90* alone, albeit the phosphorus content in lumbar vertebrae tended to be higher compared to OVX (not significant; Fig. 3). The probiotic in the diet significantly increased the fecal counts of lactobacilli after 16 weeks compared to OVX (Table 5) without affecting the other genera. The pH in the cecal digesta tended to be reduced ($p < 0.1$) (Table 6).

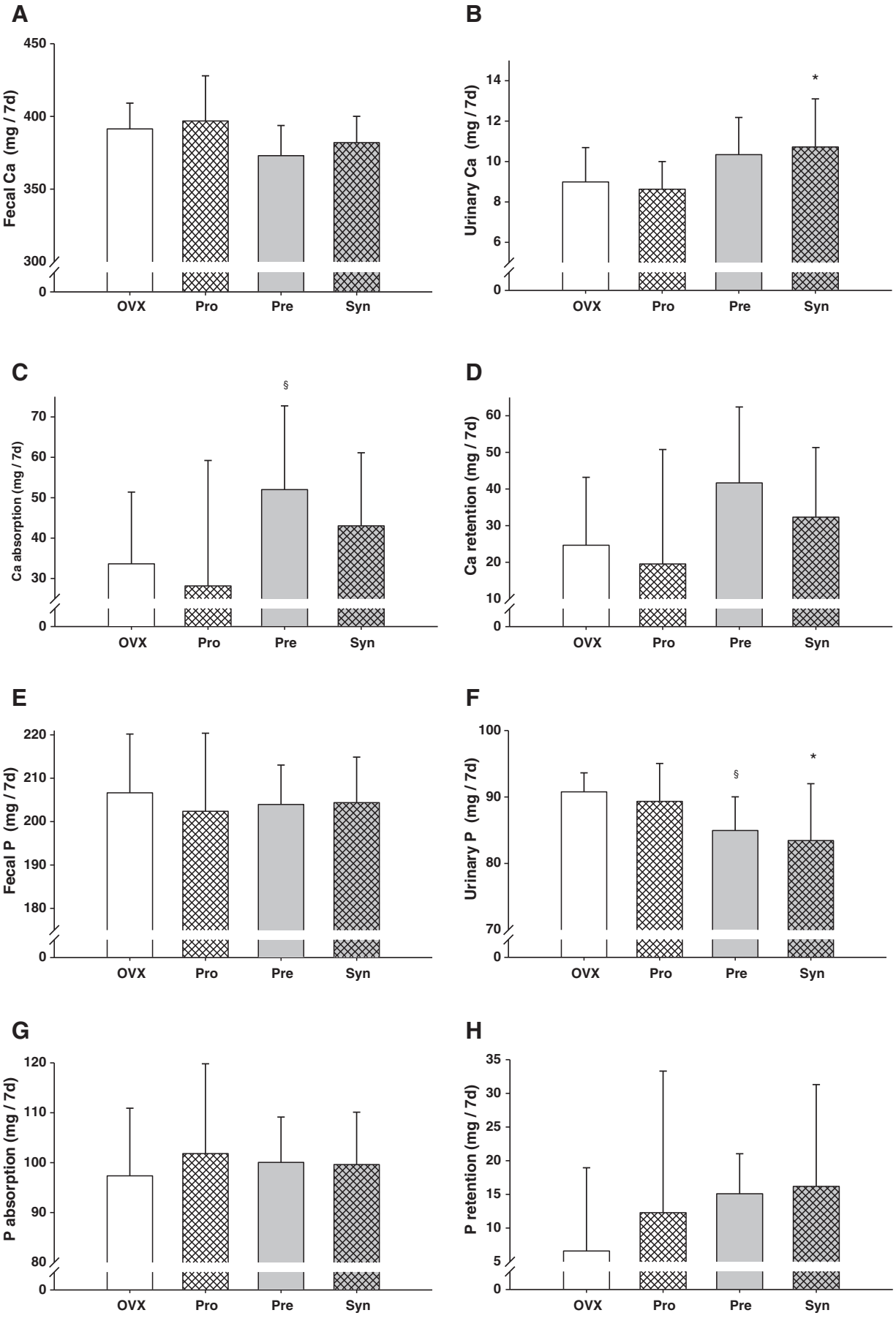
3.3. Effects of prebiotics

In the short-term, after 6 weeks of intervention, animals on prebiotics (PRE) had a significantly higher fecal dry mass (5.34 ± 0.28 g/7 days versus 4.97 ± 0.28 g/7 days; $p < 0.05$), and significantly lower urinary phosphorus than the ovariectomized control animals (OVX), whereas no short-term effects were seen on calcium absorption, urinary excretion or retention (data not shown). In the long term, after 16 weeks, calcium absorption tended to be higher and urinary phosphorus to be lower than that of OVX (Fig. 2). Bone mineral content (Fig. 3) and trabecular structure in the tibia (Table 4) were not affected with the exception of a tendency towards an increase in phosphorus content in lumbar vertebrae (Fig. 3). Prebiotics significantly increased the cecum digesta content (Table 6) and decreased the pH in the digesta of cecum (Table 6) and colon (Table 6). Repeated microbial analyses have shown a significant increase of bifidobacteria in animals consuming prebiotics (PRE and SYN) compared to OVX after 6 weeks (Table 5). This increase, however, did not persist until the 16th week (Table 5). In the long-term *Bacteroides* were significantly higher in prebiotics-fed animals (PRE and SYN) than in OVX. At that time a rise of lactobacilli was seen in the PRE (but not in the SYN) group (Table 5).

3.4. Effects of synbiotics

In the short-term, 6 weeks after intervention, fecal dry mass was significantly higher in rats on diets containing synbiotics (SYN) with 5.32 ± 0.28 g/7 days compared to OVX with 4.97 ± 0.28 g/7 days ($p < 0.05$). Urinary calcium tended to be higher but there was no significant effect on calcium absorption or retention (data not shown). Urinary phosphorus was significantly lower compared to OVX (data not shown). After 16 weeks intervention calcium absorption was 27% higher and retention was 31% higher than those in OVX (Fig. 2) but the difference failed to reach significance ($p > 0.1$). Fecal phosphorus excretion and apparent absorption (Fig. 2) were not affected by synbiotics, whereas urinary phosphorus was lower ($p < 0.05$) than in OVX. Feeding synbiotics only slightly affected femur calcium (Fig. 3) but significantly increased the calcium and phosphorus content of lumbar vertebrae (Fig. 3). Synbiotics slightly improved trabecular bone area (Tb.Ar/T.Ar) compared to OVX, but without significance for this or any other of the

Fig. 2. Effect of probiotic (*L. acidophilus NCC90*, PRO), prebiotic (oligofructose + acacia gum, PRE) and synbiotic (probiotic + prebiotic, SYN) on fecal calcium (A), urinary calcium (B), calcium absorption (C), calcium retention (D), fecal phosphorus (E), urinary phosphorus (F), phosphorus absorption (G) and phosphorus retention (H) in ovariectomized rats after 16 weeks on experimental diets. Values are means ± SD, $n = 15–16$. *Significant difference ($p < 0.05$); §tendency ($p < 0.1$) compared to the ovariectomized control (OVX) as assessed by Dunnett's test.


A
Fecal Ca (mg / 7d)
450
400
350
300
0
OVX
Pro
Pre
Syn
B
Urinary Ca (mg / 7d)
14
12
10
8
6
0
*
OVX
Pro
Pre
Syn
C
Ca absorption (mg / 7d)
70
60
50
40
30
0
§
OVX
Pro
Pre
Syn
D
Ca retention (mg / 7d)
60
50
40
30
20
10
0
OVX
Pro
Pre
Syn
E
Fecal P (mg / 7d)
220
210
200
190
180
0
OVX
Pro
Pre
Syn
F
Urinary P (mg / 7d)
100
90
80
70
0
§
*
OVX
Pro
Pre
Syn
G
P absorption (mg / 7d)
120
110
100
90
80
0
OVX
Pro
Pre
Syn
H
P retention (mg / 7d)
35
30
25
20
15
10
5
0
OVX
Pro
Pre
Syn

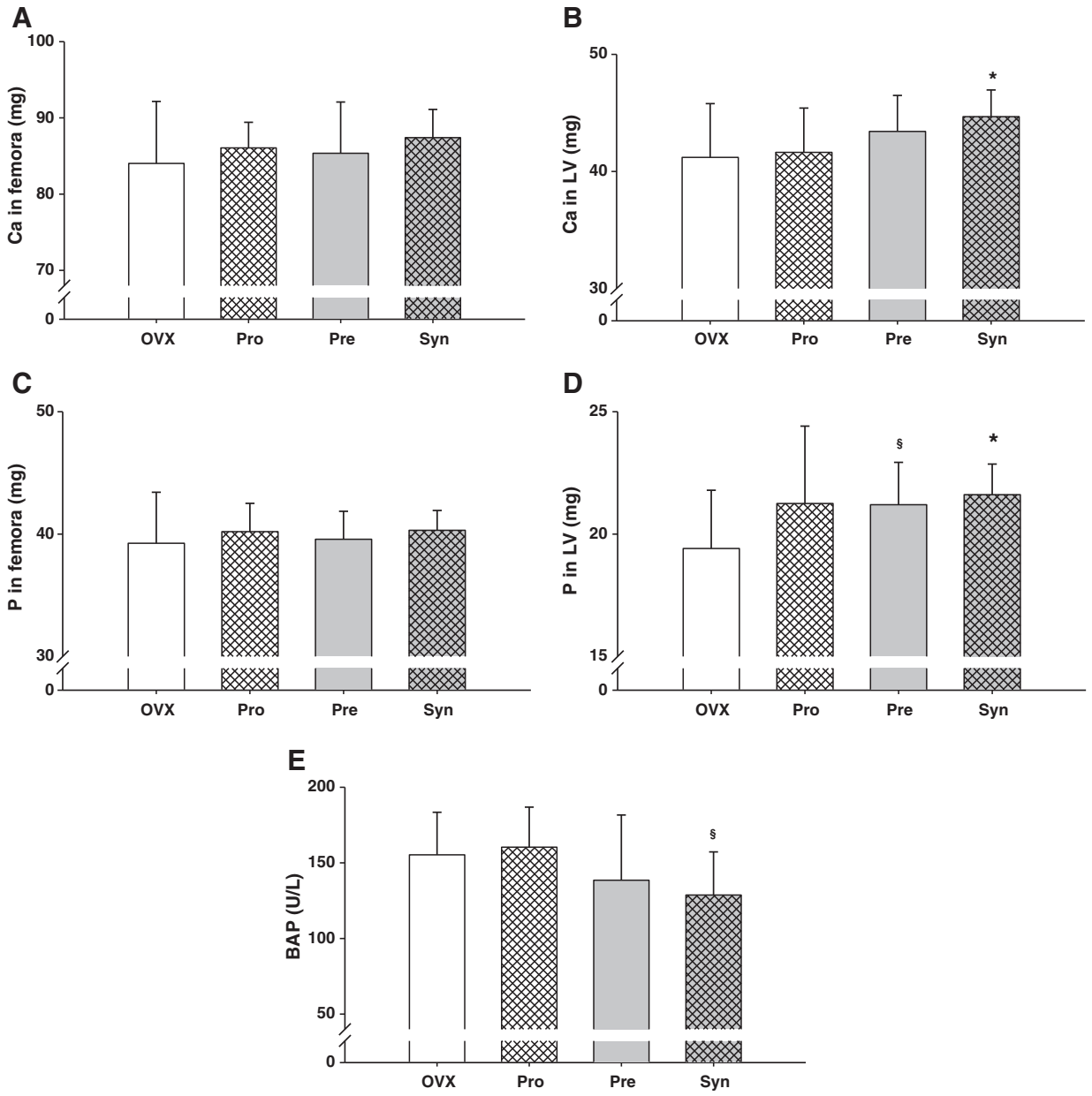


Fig. 3. Effect of probiotic (*L. acidophilus NCC90*, PRO), prebiotic (oligofructose + acacia gum, PRE) and synbiotic (probiotic + prebiotic, SYN) on calcium content in femora (A) and lumbar vertebrae (LV) (B), phosphorus content in femora (C) and in lumbar vertebrae (D) and bone alkaline phosphatase (BAP) (E) in ovariectomized rats after 16 weeks on experimental diets. *Significant difference ($p < 0.05$); §tendency ($p < 0.1$) compared to the ovariectomized control (OVX) as assessed by Dunnett's test.

histomorphometric parameters (Table 4). BAP was lowest after SYN and tended to be lower compared to OVX (Fig. 3). Synbiotics decreased the pH in the digesta of jejunum ($p < 0.1$), cecum ($p < 0.05$) and colon ($p < 0.1$), and slightly but not significantly increased digesta content in the cecum (Table 6). Jejunum weight was lower ($p < 0.05$) and colon weight was higher ($p < 0.1$) in the SYN compared to the OVX group (Table 6). Feeding synbiotics for 6 weeks significantly increased the fecal counts of bifidobacteria which were highest of all the groups (Table 5). In the long-term, a significant increase of *Bacteroides* was observed after 16 weeks (SYN versus OVX; Table 5).

3.5. Correlations between gut ecology and bone parameters in all ovariectomized groups

The correlations between bone mineral (mg calcium and mg phosphorus per bone in the femur and lumbar vertebrae) as the primary outcome and the pH of digesta were not significant. In contrast to this gut weight (indicating intestinal absorption surface), and weight of cecum and colon digesta (indicating luminal microbial mass) showed significant correlations with bone mineral content (Table 7). BAP was negatively associated with bone mineral content (Table 7).

4. Discussion

4.1. Validation of the experimental model

The effects of probiotics, prebiotics and synbiotics on mineral metabolism were investigated in adult virgin ovariectomized rats, an accepted animal model to test dietary effects in postmenopausal osteoporosis [3,23]. Indeed ovariectomy resulted in the expected alterations of bone parameters (Table 3. The increase in body weight (Table 3) is in line with the increase in body weight, subcutaneous and perineal

Table 4
Effect of diets containing probiotics, prebiotics, and synbiotics on bone histomorphometric variables in ovariectomized rats.

		OVX	PRO	PRE	SYN
Tb.Ar/T.Ar	(%)	12.25 ± 2.81	12.81 ± 4.91	13.99 ± 4.43	13.60 ± 5.34
Tb.N	*n*	28.2 ± 10.26	29.00 ± 8.08	28.38 ± 9.16	24.44 ± 7.62
Tb.Pm	mm	29.52 ± 5.64	29.25 ± 5.10	30.18 ± 6.69	27.54 ± 7.15
Tb.Th	mm	0.020 ± 0.002	0.020 ± 0.004	0.020 ± 0.003	0.021 ± 0.003
TBPF	mm^{-1}	25.32 ± 4.40	23.79 ± 7.06	22.62 ± 7.96	25.07 ± 9.41
C.Th	mm	0.38 ± 0.16	0.35 ± 0.08	0.37 ± 0.06	0.38 ± 0.14
EpB	mm	0.093 ± 0.012	0.097 ± 0.006	0.087 ± 0.013	0.089 ± 0.013
Tb.BP	*n*	134.07 ± 27.48	127.56 ± 33.15	132.31 ± 30.78	127.00 ± 34.75

Means ± SD after 16 weeks intervention, $n = 15–16$. OVX = ovariectomized control rats;
PRO = OVX + Probiotics (1–5*10^6 cfu of *L. acidophilus NCC90*/g) mixed with yoghurt;
PRE = OVX + Prebiotics (fructooligosaccharide, Raftilose P95® + acacia gum (50:50);
SYN = OVX + Synbiotics (PRO + PRE).
Variables are trabecular bone area (Tb.Ar/T.Ar), trabecular number (Tb.N), trabecular perimeter (Tb.Pm), mean trabecular thickness (Tb.Th), and trabecular bone pattern factor (TBPf), which indicates a loss of connectivity, cortical bone thickness (C.Th), epiphyseal breadth (EpB), trabecular branch points (Tb.BP).
There were no significant differences from OVX ($p > 0.05$) in the variables following Dunnett's procedure.

adipose tissue observed by Cox-York et al. [27]. Interestingly, pH of the jejunal digesta was increased by ovariectomy (Table 3). This is in agreement with the findings of Cox-York et al. [27] who observed a reduction of short chain fatty acids and alterations of the intestinal microbiota by ovariectomy.

4.2. Effects of probiotics

Until now little is known on effects of potentially probiotic bacteria on bone density and mineral metabolism [21]. Some specific probiotics reduced aging-induced bone loss in senescence-accelerated mice [28]; others improved bone density in mice [29] or increased cortical bone mineral content in chicken [30]. The specific probiotic (*L. acidophilus NCC90*) alone did not show a significant effect on mineral balance data (in contrast to PRE or SYN) in spite of its capacity to slightly lower the pH in cecum digesta. Obviously, *L. acidophilus NCC90* did not stimulate calcium uptake, comparable with *Bifidobacterium infantis* [31], while others (*Lactobacillus salivarius*) did [31]. Accordingly either the strain was not effective or the amount given was too low and the rise of lactobacilli in the gut (Table 5) too small to affect mineral absorption. The more consistent effects in SYN compared to PRE, however, indicate existence of a probiotic effect. This, however, may be bound to sufficient fermentable substrate reaching the lower gastrointestinal tract.

4.3. Effects of prebiotics

Increased fecal dry mass after 6 weeks in the prebiotic group (PRE) together with lower pH in cecal and colonic digesta and higher weight of cecum content after 16 weeks intervention indicate enhanced luminal fermentation and bacterial mass as well as trophic effects on the intestine and confirm effects described previously for oligofructose [12]. The mixture of oligofructose + acacia gum tended to enhance calcium absorption and nominally, but not significantly, retention in the long-term. An enhancement of calcium absorption and retention was shown for various other prebiotics [10–12]. In adult ovariectomized rats the stimulating effect of oligofructose on calcium absorption depended on the level of dietary calcium: at a lower level of calcium (5 g Ca /kg diet) 100 g oligofructose/kg diet was required to stimulate calcium absorption in adult rats, while 50 and 25 g/kg diet proved to be insufficient [23]. In the present study just 25 g prebiotics/kg was sufficient to elevate calcium absorption, showing that the mixture of oligofructose + acacia gum was effective. One explanation may be that the incorporation of slowly fermented acacia gum into our PRE mixture extended the duration of fermentation. According to in vitro data the fermentation profile was improved after the administration of a prebiotic mixture containing acacia gum compared to FOS alone [32]. Acacia gum optimized the microbial activity as indicated by a more uniform distribution of fermentation products along the

Table 5
Effects of diets containing probiotics, prebiotics, or synbiotics on fecal bacterial counts in ovariectomized rats after 6 and 16 weeks intervention.

	OVX	PRO	PRE	SYN
	log (cfu/g of feces)			
Week 6				
Enterobacteriaceae	5.79 ± 0.96	5.89 ± 0.88	5.94 ± 0.84	5.73 ± 1.00
Bacteroides	7.05 ± 0.20	6.55 ± 0.60	7,18 ± 1.04	6.97 ± 1.16
Lactobacilli	7.48 ± 0.80	7.42 ± 0.52	7.70 ± 0.52	7.71 ± 0.72
Bifidobacteria	3.35 ± 0.16	3.30 ± 0.04	4.73 ± 1.92[a]	6.15 ± 2.04[a]
Week 16				
Enterobacteriaceae	5.53 ± 0.72	5.93 ± 0.76	5.89 ± 0.72	5.93 ± 0.84
Bacteroides	7.33 ± 0.48	7.42 ± 0.44	7.90 ± 0.64[a]	8.25 ± 0.56[a]
Lactobacilli	7.75 ± 0.44	8.20 ± 0.40[a]	8.19 ± 0.36[a]	7.88 ± 0.48
Bifidobacteria	5.28 ± 2.00	4.86 ± 2.08	4.03 ± 1.60	5.29 ± 2.08

Means ± SD, $n = 16$. OVX = ovariectomized control rats;
PRO = OVX + Probiotics (1–5*10^6 cfu of *L. acidophilus NCC90*/g) mixed with yoghurt;
PRE = OVX + Prebiotics (fructooligosaccharides, Raftilose P95® + acacia gum (50:50);
SYN = OVX + Synbiotics (PRO + PRE).
[a] Significantly different from OVX control with $p < 0.05$ (Dunnett's procedure).

Table 6
Effects of diets containing probiotics, prebiotics, and synbiotics on body weight and parameters of gut ecology in ovariectomized rats.

	OVX	PRO	PRE	SYN
Body weight (g)				
Week 0	169.56 ± 7.69	169.05 ± 8.09	169.85 ± 7.64	170.67 ± 6.58
Week 16	184.39 ± 3.54	182.39 ± 5.19	179.60 ± 8.25[b]	183.04 ± 5.95
Uterus weight (g)				
Week 16	0.085 ± 0.020	0.088 ± 0.017	0.095 ± 0.020	0.093 ± 0.011
Bowel weight (g)				
Jejunum	3.52 ± 0.48	3.64 ± 0.52	3.14 ± 0.50	2.99 ± 0.57[a]
Cecum	0.66 ± 0.14	0.67 ± 0.14	0.73 ± 0.16	0.67 ± 0.14
Colon	0.56 ± 0.09	0.53 ± 0.11	0.60 ± 0.09	0.64 ± 0.07[b]
Digesta weight (g)				
Jejunum	1.26 ± 0.20	1.17 ± 0.32	1.29 ± 0.53	1.42 ± 0.54
Cecum	2.69 ± 0.36	2.63 ± 0.44	3.60 ± 1.28[a]	3.28 ± 0.87
Colon	0.90 ± 0.24	1.00 ± 0.20	0.99 ± 0.19	0.97 ± 0.27
Digesta pH				
Jejunum	7.69 ± 0.45	7.49 ± 0.35	7.48 ± 0.40	7.34 ± 0.43[b]
Cecum	8.00 ± 0.29	7.75 ± 0.26[b]	7.37 ± 0.37[a]	7.51 ± 0.29[a]
Colon	8.25 ± 0.29	8.09 ± 0.34	7.92 ± 0.22[a]	8.03 ± 0.23[b]

Means ± SD after 16 weeks intervention, $n = 16$. OVX = ovariectomized control rats.
PRO = OVX + Probiotics (1–5*10^6 cfu of *L. acidophilus NCC90*/g) mixed with yoghurt;
PRE = OVX + Prebiotics (fructooligosaccharides, Raftilose P95® + acacia gum (50:50);
SYN = OVX + Synbiotics (PRO + PRE).
[a] Significantly different from OVX control with $p < 0.05$ (Dunnett's procedure).
[b] Different from OVX control with $p < 0.10$ (Dunnett's procedure).

Table 7
Pearson correlation coefficients (r) between femoral and vertebral calcium or phosphorus content and intestinal parameters or BAP in ovariectomized rats (including the groups OVX control, PRO, PRE and SYN) ($n = 64$).

	Femur		Lumbar vertebra	
	Total Ca (mg)	Total P (mg)	Total Ca (mg)	Total P (mg)
Bowel weight (g)				
Jejunum	0.21	0.43**	0.09	0.09
Cecum	0.24	0.34**	0.22	0.24
Colon	0.03	−0.06	0.09	0.06
Digesta weight (g)				
Jejunum	−0.20	−0.33**	−0.17	−0.03
Cecum	0.37**	0.29*	0.29*	0.17
Colon	0.22	0.20	0.27*	0.29*
Digesta pH				
Jejunum	−0.08	−0.03	−0.16	−0.16
Cecum	0.02	0.03	−0.03	−0.13
Colon	0.17	0.16	0.04	−0.04
Plasma				
BAP (U/L)	−0.23	−0.30*	−0.43**	−0.27*

*/**Significant correlation with $p < 0.05$/$p < 0.01$.

ascending, transverse and descending "simulated colon" in the TWINSHIME model [32].

Metabolic mineral balances, even though repeatedly investigated over time, just give a snapshot of the actual physiological occurrence and do not reflect the long-term outcomes. Furthermore, methods may differ in sensitivity with respect to specific questions, like short-term vs. long-term effects, or effects after a new steady-state has been reached. In contrast to reports by Abrams (17) and Whisner et al. [33] the apparent calcium absorption did not show a benefit in the short-term, which may be surprising. However, these studies [17,33] were done in growing human subjects, while the adult rats in our study had to adapt to dietary changes (calcium content, phosphorus content) and to a new hormonal state after ovariectomy.

The stimulation of calcium absorption after 16 weeks at the end of the study was in concordance with the alterations in intestinal ecology, mainly the reduction of pH of the cecal and colonic contents, and the increase of cecum contents. However, the increment of calcium absorption was not reflected in a higher calcium content of the axial or appendicular skeleton. It was not reflected either in a rise of tibia trabecular area, which was the case in a prior trial, in which oligofructose alone was applied at the same dose [23].

4.4. Effects of synbiotics

The combined application of a probiotic with a prebiotic has synergistic effects, if the prebiotic is preferentially fermented by the administered probiotic bacterial strain or otherwise favoring the strain with entailing effects in the intestine and on mineral metabolism. We observed that the weight of the intestinal segments (Table 6) and the bacterial composition in the feces (Table 5) were affected in rats on synbiotics. Indeed one may have expected higher counts of lactobacilli in the synbiotic group as well, but obviously there were indirect effects by the prebiotic within the synbiotic group that affected the impact of the probiotic. Bifidobacteria and lactic acid bacteria are able to ferment FOS [34]. After the combined administration of oligofructose and *L. acidophilus 74-2* a higher content of bifidobacteria, but not lactobacilli, was observed in vitro in a human intestinal ecosystem simulator [35], which goes along with our short-term observations. On the other hand, higher counts of fecal lactobacilli have been reported if rats consumed FOS of different chain length [36]. Obviously probiotics affect the growth of other strains of the habitual microbiota, and the chain length of prebiotics has an impact on the composition of the cecal, colonic or fecal microflora. Obviously synbiotics affected the composition of the gut microbiota and thereby helped to better exploit the supplied food with respect to calcium and phosphorus leading to a higher bone mineral content. Calcium balance after 6 weeks did not reflect the long-term outcomes for the skeleton, whereas it did so after 16 weeks. However, balances are just snapshots and effects can be small and non-significant even though repeatedly observed, while long-term or cumulative effects measured in bone are significant [11,23]. Therefore, outcomes based on analyses of bone itself are considered as more meaningful than single balances.

The loss of bone mineral content after ovariectomy was slightly alleviated in the femora and significantly reduced in the axial skeleton by synbiotics. The more pronounced bone mineralization after synbiotics compared to the prebiotics thus can in part be explained by the higher number of fecal bifidobacteria (after 6 weeks) but not of lactobacilli (after 16 weeks). Interestingly, *Bacteroides* counts were highest in rats on synbiotics. This is in contrast to higher counts of bifidobacteria but lower counts of *Bacteroides* in fecal samples of overweight humans after consuming trans-galactooligosaccharides [37]. Fitting to our findings acacia gum increased fecal bifidobacteria, lactobacilli and *Bacteroides* in healthy human volunteers [25]. Our findings of highest *Bacteroides* counts in the group with the highest bone mineral content are in agreement with findings by Whisner et al. [33]. They observed a positive correlation between a stimulated calcium absorption and percentage of *Bacteroides* after prebiotics (soluble maize fiber). *Bacteroides* modulate expression of host genes, including those involved in nutrient absorption and mucosal barrier fortification, and thereby can affect the host's immune system [38,39].

The enhanced mineral retention in rats on diets containing prebiotics (PRE and SYN), although statistically not significant, and the coherent changes in gut ecology are in concordance with the underlying mechanisms that are discussed for prebiotics and mineral metabolism [10,12,13,14,40,41]. The rise of bifidobacteria, which we observed in the PRE and SYN group, in spite of being a transient effect, obviously contributed to raise acidity and mineral solubility and absorption. Lactobacilli were higher in the PRO group, but not in the SYN group, although these animals had received the same probiotic yoghurt. The SYN group had increased counts of *Bacteroides*, as did the PRE group. The latter also showed a rise in lactobacilli without having consumed them during intervention. Obviously the prebiotic substrate containing acacia gum matched the demands of the innate lactobacilli of the PRE group. Our observations correspond to findings in humans [25]. It seems that specific bacteria in the diet and their combination with a specific prebiotic substance initiate a shift in the innate microbiota to a composition that is more favorable for the host's skeleton than either dietary intervention (PRO or PRE) alone.

BAP as an indicator for osteoblast proliferation and activity or for increased bone turnover is generally increased during growth and reduced when bone formation is hampered or bone turnover is slowed down. BAP was significantly reduced after ovariectomy (Table 3), presumably through dysregulation of osteoblast and osteoclast formation [42]. None of the experimental diets reversed this effect. In contrast, BAP was further decreased after synbiotics (Fig. 3E), indicating a diminished bone turnover and thus a diminished bone resorption as was observed after intervention with *Lactobacillus reuteri* [43]. Our assumption is compatible with a reduction of bone turnover in postmenopausal women after hormone replacement therapy [44] or after consumption of fermented milk supplemented with calcium [45] and may be based on a reduction of the inflammatory level. Nevertheless, bone markers are difficult to interpret if they had reached a new steady-state, and results should not be over-interpreted until being confirmed.

Although we did not analyze parameters of inflammation or bone resorption, there is evidence for intersections between osteoporosis and inflammation [43,46]. Inflammatory cytokines are elevated in estrogen deficiency osteoporosis [46], and a lack of estrogens on its own was associated with higher values for TNF-α [43]. This cytokine stimulates osteoclast differentiation and bone resorption [47] and thereby

also mediates OVX-induced osteoporosis. Some probiotic strains have been found to reduce inflammation and inflammatory cytokines [48–50], and thus have therapeutic potential with respect to bone health. In a mouse model *L. reuteri* 6475 prevented ovariectomy-induced bone loss [43]. The probiotic strain we used obviously has no bone mineral protective potential on its own, but it has when given together with the prebiotic combination of oligofructose + acacia gum. Oligofructose has an anti-inflammatory potential on its own, as we observed in our laboratory in Caco-2 cells [51], and short chain fatty acids, particularly butyrate, products of microbial carbohydrate metabolism, were also shown to exert anti-inflammatory effects [52]. Indeed butyrate concentration was higher when a specific lactobacillus strain (*L. acidophilus DSM 20079*) was cultivated in the presence of inulin compared to glucose [53]. These findings offer bone health-favoring mechanisms by probiotics and prebiotics that rely at least in part on effects independent of luminal acidity, solubility of minerals or absorption surface.

The results of correlations between parameters of gut ecology and those of bone (Table 7) indeed suggest that lowering pH has less impact on bone mineralization than the mass of digesta in the gut lumen and the mass of intestinal tissue. Luminal bowel content in the lower gastrointestinal tract is mainly composed of microbes. They are known to release growth factors and to exert trophic effects on the intestine [54,11,12], which may explain the increase in intestinal tissue. In fact the increase in bowel weight was limited to the large bowel (Table 6), where the major mass of the intestinal microbiota is found. In this context one has to bear in mind that calcium absorption occurs via two transport processes [55]. The active, transcellular and saturable one depends on vitamin D and is predominant in the proximal intestine. The passive paracellular calcium transport occurs throughout the intestine provided that calcium is present in its soluble form [55]. In the present study we investigated prebiotics (and synbiotics) and their potential to stimulate calcium absorption. They are mainly fermented in the large intestine and exert their trophic and solubility effects via modulating microbiota in this intestinal section. Both, a) improved mineral absorption (by stimulated fermentation and increased absorptive surface) and b) possibly lower inflammatory level (by improved intestinal barrier function and anti-inflammatory effects of microbiota, of their metabolites, including SCFAs, and also of oligosaccharides themselves) may have contributed to the effects of synbiotics on bone.

5. Conclusion

Based on our observations we conclude that loss of bone mineral by ovariectomy was prevented mostly and significantly by a combination of the specific prebiotic (oligofructose + acacia gum) with *L. acidophilus NCC90*. The beneficial effects were associated with slightly increased microbial mass implying higher fermentation capacity, trophic signaling and anti-inflammatory effects with alteration of the microbiota, and with a tendency for increased large bowel weight and thus absorptive area. Apart from bifidobacteria and lactobacilli *Bacteroides* were involved and prevention of bone loss was obviously mediated through reduction of bone turnover.

Conflicts of interest

K.E.S., B.A., M.d.V., and Y.A. report no conflicts of interest except being part of the research team. J.S. received research funding for this study from NESTEC Lausanne, Switzerland. J.S. received fees for consulting Danone, Yakult, Dr. Fischer Health Care, Campina, Infectopharm, Merck, funds and grants for research from Campina, Chr. Hansen. Danisco, Danone, Merck, MONA, Morinaga, Mueller, Nestle, NO M, Wakunaga and Yakult for studies on probiotics, and fees as a speaker at symposia sponsored by Yakult, Danone, Nestle, Orthomol, and Merck. F.R. and D.V.B. are/were salaried employees of NESTEC Ltd.

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K.E.S.A., D.V.B., and J.S. designed the research.

K.E.S.A., B.A., F.R., and Y.A. conducted the research.

D.V.B. (NESTEC) provided essential materials (probiotic bacteria and prebiotic mixture).

K.E.S.A., B.A. and M.V. analyzed data or performed statistical analysis.

K.E.S.A. and J.S. wrote the paper.

All authors have read and approved the final article.

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